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## Toxicity Studies of Trehalose and / or Methotrexate in Mice.

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### ABSTRACT

Trehalose compound has no cytotoxic effect on naïve mice [1]. It shows protective effects in various cells against harmful stimuli such as heat, dehydration, cold, desiccation and oxidation [2] and it has antitumour effect against Erlich ascites carcinoma [3]. In this study, mice were assigned into four groups: control group, trehalose group (200 µg/mouse), methotrexate group (25 µg/mouse), trehalose plus methotrexate group, six times a day. On day 14<sup>th</sup>, mice were euthanized. Biochemical parameters such as hepatic enzymes (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase), albumin and total protein in serum have been determined. Glutathione S. transferase, catalase, total antioxidants and malondialdehyde in liver tissue, in addition to complete blood count (CBC) and histological studies of all groups were carried out. The results indicate that trehalose has no cytotoxic effect on naïve mice when compared with methotrexate as chemotherapeutic agent only and trehalose may be considered as a novel drug.

**Keywords:** *Trehalose, Methotrexate, Cytotoxic effect*

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## INTRODUCTION

Trehalose is a natural disaccharide found in organisms ranging from bacteria to plants, including yeasts, fungi and vertebrates. It protects cells against various environmental stresses [1]. Whereas some of the protective effects may be explained by its chemical chaperone properties, its actions are largely unknown except that it induces autophagy in an mTOR-independent manner which is conserved catabolic mechanism in which the unwanted organelles and misfolded proteins are delivered to lysosome for degradation and the final metabolic products can be recycled as nutrient for keeping cell homeostasis [10]. It shows protective effects in various cells against harmful stimuli such as heat, dehydration, cold, desiccation and oxidation [2].

The aim of the present study was to investigate the cytotoxic effect of trehalose and/or methotrexate. The criteria to assess the protective effect of methotrexate and/or trehalose include:

- 1) measurement of the oxidative stress by assessing the levels of malondialdehyde (MDA), catalase, glutathione -S- transferase (GST) and total antioxidant capacity of liver tissues homogenate and liver functions (transaminases, alkaline phosphatase, total protein and albumin) of all mice groups to reflect changes occurring in the liver of control and injected mice groups.
- 2) complete blood count (CBC) of untreated and treated groups to show the effect of tumor and treatment on blood cells.
- 3) histological studies of liver tissues of control and injected mice to show the efficacy of the drugs on liver cells.

## MATERIALS AND METHODS

### Chemicals

*Trehalose*, *methotrexate*, ferric tripyridyltriazine ( $\text{Fe}^{\text{III}}$ -TPTZ) and other chemical grade have been purchased from Sigma Aldrich company (St. Louis Mo., U.S.A).

### Experimental animals

Female Swiss albino mice, weighing 18-22 g were used as experimental animals throughout the study. Mice were purchased from National Cancer Institute, Cairo University, Egypt. The animals were handled at laboratory standard experimental conditions (temperature  $23^{\circ}\text{C}\pm 2^{\circ}\text{C}$ , relative humidity 55%  $\pm$  5% balanced diet and free access to water) and were let for about one week before experiments to adapt the laboratory conditions. Animals' procedures were consistent with the guidelines of Ethics by Public Health Guide for the Care and Use of Laboratory Animals [4].

### Experimental design

Mice were divided into four groups, each group included 15 mice as follows: Group I: (Negative control group) Mice have been injected with normal saline (0.9% w/v, 200  $\mu\text{L}$ /mouse intraperitoneally (i.p) for six times day after day. Group II: (Trehalose injected group) Mice have been injected with trehalose (from Sigma-Aldrich Co. USA) (200  $\mu\text{g}$ /mouse, 200  $\mu\text{L}$ /mouse (i.p) for six times day after day [3]. Group III: (Methotrexate injected group) Mice have been injected with methotrexate (from Sigma-Aldrich Co. USA) (25  $\mu\text{g}$ /mouse, 200  $\mu\text{L}$ /mouse (i.p) for six times day after day [3][5]. Group IV: (Trehalose and methotrexate injected group) Mice have been injected with trehalose (200  $\mu\text{g}$ /mouse, 200  $\mu\text{L}$ /mouse (i.p)) and methotrexate (25  $\mu\text{g}$ /mouse, 200  $\mu\text{L}$ /mouse (i.p)) for six times day after day.

### Sampling

Mice of all groups were euthanized, blood specimens were collected and assayed for biochemical parameters and complete blood count. Accurately part of weighed liver tissues were homogenized in phosphate buffer pH (7.4) and frozen at  $-20^{\circ}\text{C}$  till the enzyme activities were determined to indicate the state of the liver of control and injected groups and finally, part of weighed liver tissues were used for histological studies.

### Biochemical parameters assays

**Parameter assays-** AST and ALT were estimated through measuring oxaloacetate and pyruvate produced respectively [3]. ALP was measured as a liberated phenol in the presence of 4-aminoantipyrine and sodium arsenate as a blocking agent and potassium ferricyanide as a color producing complex. The developed color measured at 510 nm [3]. Total protein was estimated using Biuret test in which an intense violet-blue complex is formed with copper salts in an alkaline medium [3]. Iodide is included as an antioxidant and bovine serum albumin. The developed color was measured at 595 nm. Albumin was estimated in the presence of bromocresol green at a slightly acidic pH, produces a colour change from yellow to green and green to blue, measured at 630 [3]. Enzyme activities were evaluated using end point assay method. GST: the formation of the adduct due to conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB); the absorbance was observed at 340 nm [3]. Catalase: 3 mL buffered H<sub>2</sub>O<sub>2</sub> was, mixed by the sample was read for 1min at 250 nm[3]. MDA was estimated and measured by colorimetric at 530 nm [3]. TAC: At low pH, when a ferric tripyridyltriazine [Fe(III)-TPTZ] complex was reduced to the ferrous form (Fe II), an intense blue colour with an absorption maximum at 593 nm developed and hence colourformation exhibited the reducing ability of the sample according to El-Magdet *al.*,2017 [3].

### Statistical analysis

One-way analysis of variance (ANOVA) was used to assess significant differences among treated groups and control. The Tukey Test was used to compare all groups with each other and showed the significant effect of treatment. The criterion for statistical significance was set at \* means  $P \leq 0.05$ , (GraphPad Prism Software).

## RESULTS AND DISCUSSION

Efforts have been directed to find a suitable natural neoadjuvant drug to increase efficacy of antitumor agents with low cytotoxicity (**Fig.1 & 2**). Our results showed that trehalose has no toxic effect on liver function tests which is in agreement with El-Magdet *al.*,2017 [3] who demonstrated that trehalose shows antitumour efficiency and in agreement with Kanget *al.*, 2014 [6] who demonstrated that trehalose shows protective effects in various cells against harmful stimuli such as heat, dehydration, cold, desiccation and oxidation. The protective effects of trehalose has been demonstrated in neurodegenerative diseases, such as Alzheimer's disease, progressive supranuclear palsy and cortico-basal degeneration. It has also been showed that methotrexate at low dose has no toxic effect on liver function tests [7].

Our data showed that intraperitoneal administration of trehalose improve total antioxidant capacity level ( $\mu\text{mol/g}$  tissue), catalase enzyme activity (mmole/min/mg protein), Glutathione-S transferase (GST) ( $\mu\text{mole/min/mg}$  protein) activity and lipid peroxidation products (MDA) (nmol/g tissue) of the liver compared to control group (**Fig. 3**). El-Magdet *al.*, 2017 [3] reported the beneficial effects of trehalose on liver by reducing extent of oxidative stress. It was reported that reactive oxygen species (ROS) are probable mediators of cytotoxicity [8].

Administration of trehalose only and trehalose plus methotrexate maintained normal values of hemoglobin but Hb showed significant decrease  $P \leq 0.05$  in methotrexate injected group when compared to the normal animals. There is no significant change in Hb of trehalose injected group in comparison to control group and that confirm that trehalose has no cytotoxic effect on naive mice. The hematological parameters as shown in table 1 indicates that trehalose may possess protective action on the hematopoietic system without inducing myelotoxicity. For 14 days, trehalose with methotrexate did not exhibit any adverse effect [3][9].

Histopathological examination of mice liver tissues of the different studied groups induced the following: normal (naïve mice) showed normal liver cells radiating from a central vein with normal hepatic cords as shown in (**Fig. 4**). Trehalose injected group showed nearly normal liver with mild congested vessels as shown in (**Fig. 5**). Methotrexate injected group showed nearly normal liver with mild dilated vessels as shown in (**Fig.6**). Trehalose plus methotrexate injected group showed nearly normal liver with mild dilated vessels as shown in (**Fig. 7**). It is clearly indicated that trehalose may possess protective action on liver tissues.

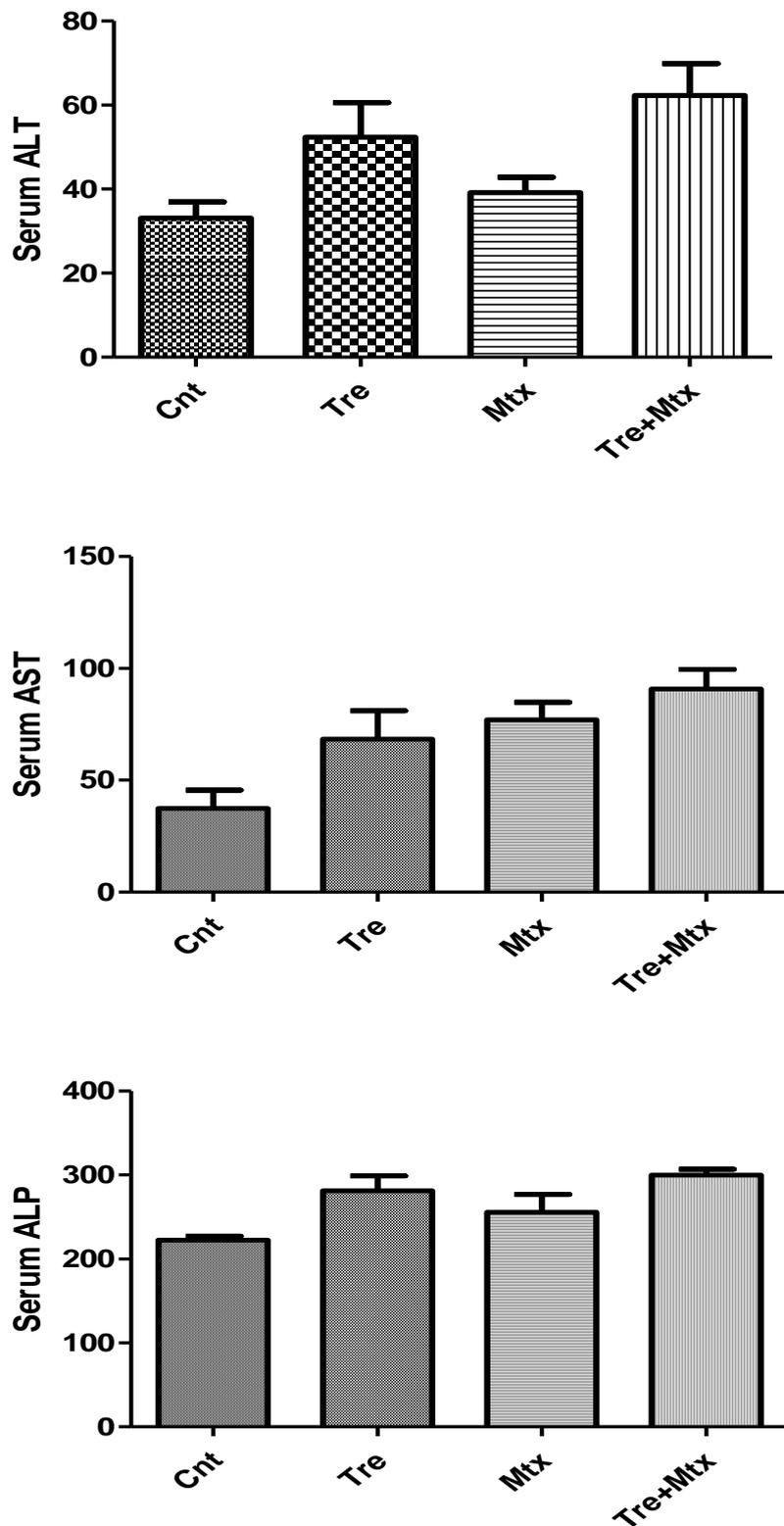


Fig 1: Effect of trehalose and /or methotrexate on serum ALT, AST and ALP in different groups.

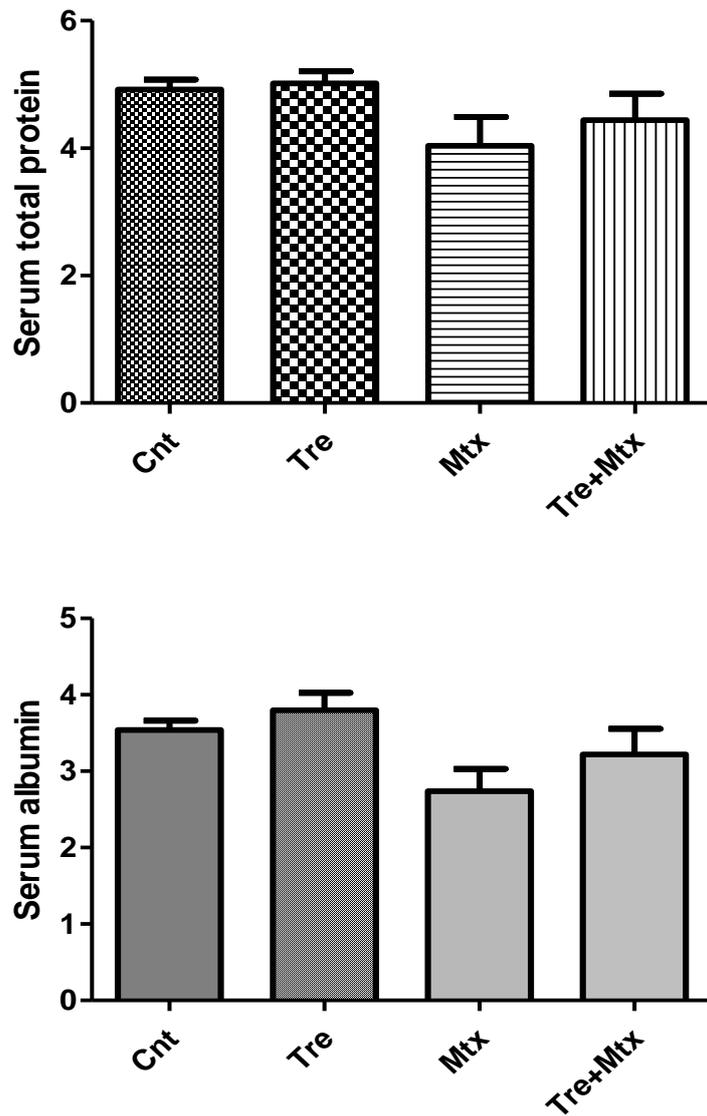
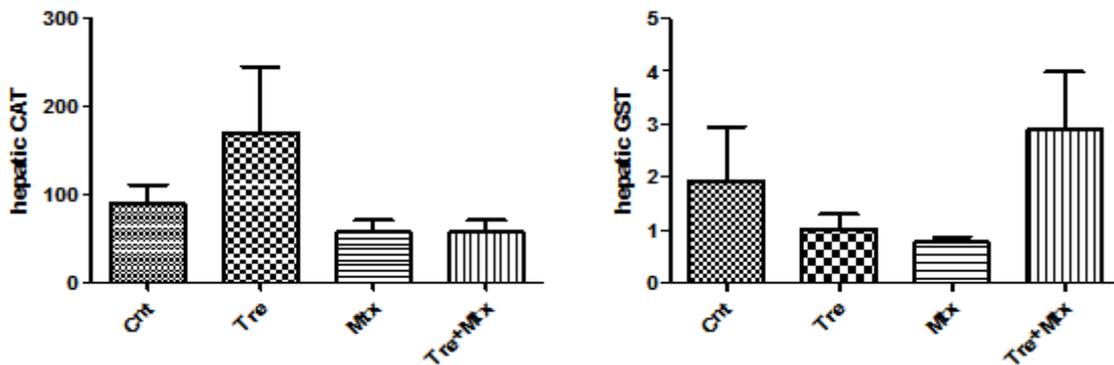


Fig 2: Effect of trehalose and /or methotrexate on serum albumin and total protein in different groups.



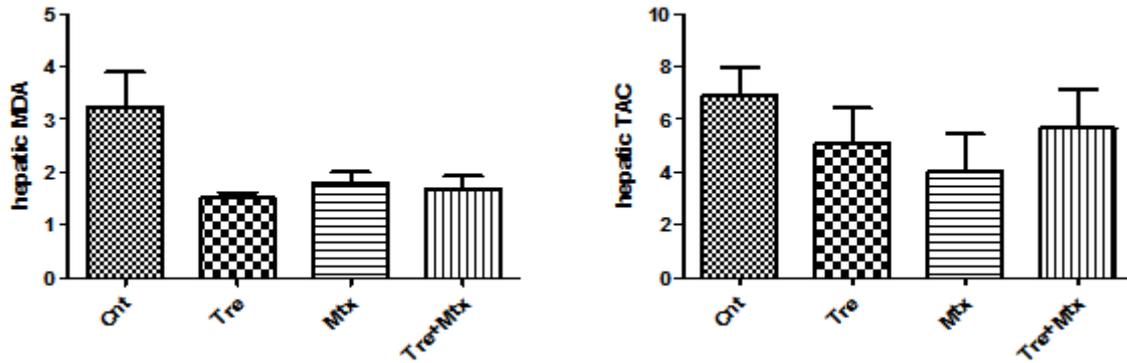


Fig 3: Effect of trehalose and /or methotrexate on liver homogenate antioxidant parameters in different groups.

Table 1: Effect of trehalose and /or methotrexate on complete blood picture (CBC) in different groups

Parameters	Cnt	TRE	MTX	TRE+MTX
Hb g/dl	11.9±0.5	11.35±0.5	8.67±0.8*	8.92±0.98
R.B.C (10 <sup>6</sup> /μl)	7.9 ± 0.1	5.5 ± 0.2	6.3 ± 0.3	4.5±0.2
W.B.C (10 <sup>3</sup> / μl )	6.6±1.6	7± 1.1	4.8±0.9	3.7±0.3
Platelets (10 <sup>6</sup> / μl )	0.8±0.02	0.7±0.04	0.7 ± 0.01	0.8±0.05
Neutrophils	1± 0.7	3.2±0.24	5±3	4.2±0.6
Lymphocytes	82±1.2	84±1.3	82.7±0.64	83.5±0.64
Monocytes	13±0.9	9.7±0.4	12±0.2	11.7±1.2

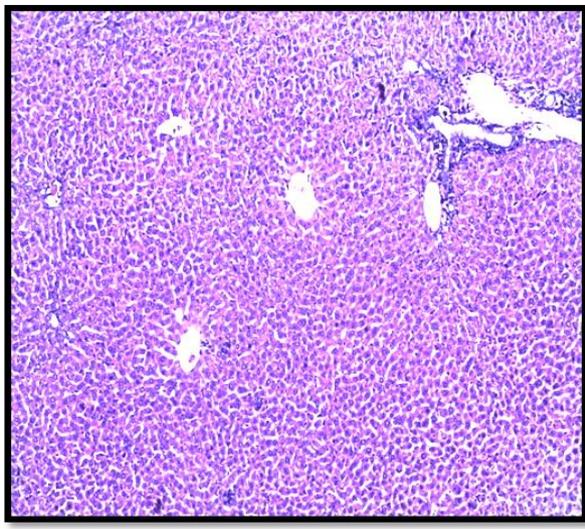


Fig 4: Photomicrographs of mice liver sections stained by H&E show normal hepatocytes, central veins and portal tract in normal mice.

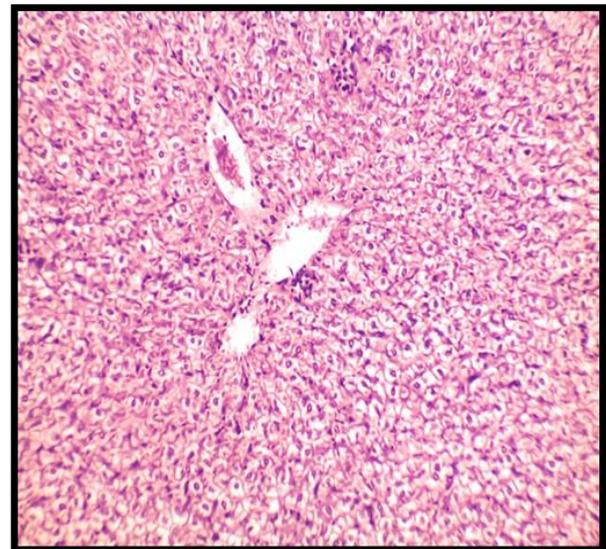
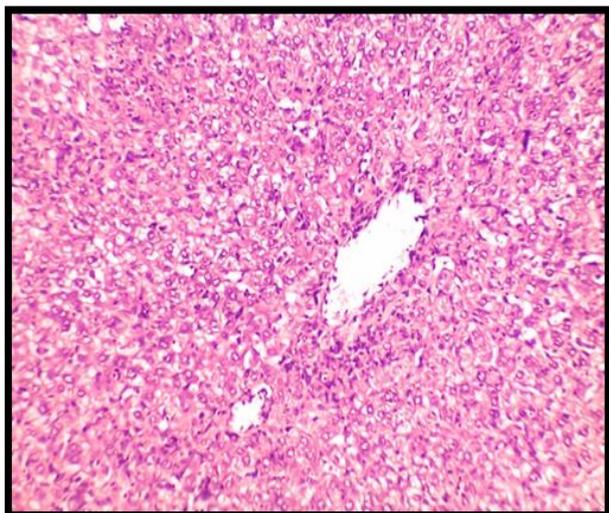
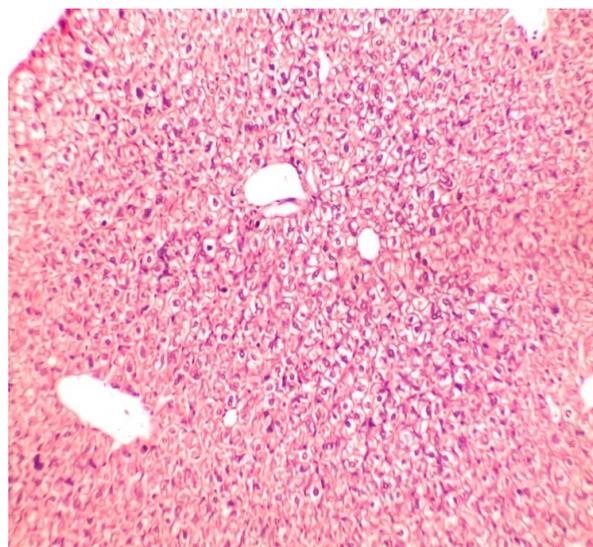


Fig 5: Photomicrographs of mice liver sections stained by H&E show nearly normal liver with mild congested vessels in TRE injected group.



**Fig 6: Photomicrographs of mice liver sections stained by H&E show normal liver with mild dilated vessels in methotrexate injected group.**



**Fig 7: Photomicrographs of mice liver sections stained by H&E show normal liver with mild dilated vessels in TRE+MTX injected group.**

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